Combination of Potato Virus *X* and Tobacco Mosaic Virus with Pepsin and Trypsin

By A. KLECZKOWSKI (Beit Memorial Research Fellow), *Rothamsted Experimental Station, Harpenden, Herts*

(*Received* 25 *February* 1944)

It is known that trypsin and pepsin combine with their substrates before causing hydrolysis (Bayliss, 1906; Northrop, 1920), but there is no evidence to show whether such combination is specific in the sense that it is restricted to substrates. Pepsin does not combine with starch, agar, kaolin or $CaSO_4$ (Northrop, 1920), but these differ so widely from proteins that this fact cannot be interpreted as indicating that substrates have a specific affinity for enzymes.

The two plant viruses, potato virus *X* and tobacco mosaic virus, seemed to afford particularly suitable material for studying this problem, for they are both nucleoproteins with rod-shaped particles and with many similar properties, but potato virus *X* is readily hydrolyzed by both pepsin and trypsin (Bawden & Pirie, 1936, 1938), whereas tobacco mosaic virus is not. However, denaturation by heat renders tobacco mosaic virus susceptible to proteolysis by both enzymes. Both viruses are readily obtained in large quantities and in the form in which they give liquid crystalline solutions. Their large size and insolubility around pH 4·0 are properties that permit easy separation from dissolved free enzymes, and their specific activities provide additional tests for changes too small to be detected by the chemical and physical tests used for studying most proteins.

Previous work has shown that although trypsin does not hydrolyze tobacco mosaic virus, mixing the enzyme with the virus leads to an immediate loss of infectivity (Caldwell, 1933; Stanley, 1934*a*; Bawden & Pirie, 1937); this phenomenon is reversible and fully infective virus can be recovered from such mixtures. Mixing trypsin with potato virus *X* also leads to an immediate drop in infectivity, but, at pH values at which trypsin is proteolytically active, this is followed by hydrolysis (Bawden & Pirie, 1936).

The action of pepsin on tobacco mosaic virus has been a subject of some controversy. The addition of the enzyme to the virus has no immediate effect, but Stanley (1934*b*) and Ross & Vinson (1937) claimed that after incubation for many days at pH 3·0 and 37° the virus lost infectivity, whereas Bawden & Pirie (1937), working with liquid crystalline virus preparations, found that pepsin had no effect, although it rapidly hydrolyzed the coagulum produced by heat denaturation of the virus. Experiments made in the course of the present work confirm Bawden & Pirie, for the virus preparations used were unaffected by as much as 10 days' incubation with 0·1 % pepsin at pH 3·0 and 37°.

In this work the ability of pepsin, trypsin and invertase to combine with the two viruses was tested, and certain effects of adsorbing the three enzymes by charcoal were also studied. Invertase was included to compare the behaviour of a non-proteolytic enzyme, which has no effect on the two viruses or on their components, with that of pepsin and trypsin.

MATERIAL AND METHODS

Liquid crystalline preparations of the viruses. Tobacco mosaic virus was isolated from sap expressed from minced leaves of infected tomato plants by the method described by Bawden & Pirie (1943), involving several successive precipitations by one-third saturation with $(NH_4)_2SO_4$, and with dilute HCl at pH 3·3.

Potato virus *X* was isolated from sap expressed from minced leaves of infected tobacco plants by a method similar to that described by Bawden & Pirie (1938). The sap was frozen overnight. After several successive precipitations by one-quarter saturation with $(NH_4)_2SO_4$ and with dilute HCl at pH 4·5 the preparations were dialyzed; the virus was then sedimented by centrifugation for 1 hr. at 40,000 r.p.m. and redissolved in water.

Enzymes. The commercial preparations of pepsin and invertase, used in this work, were obtained from The British Drug Houses Ltd. As a source of trypsin E. Merck's 'Pancreatin' was used.

Methods of testing combination. Solutions of the enzymes were mixed with solutions or suspensions of substances under test. The mixtures were centrifuged in conditions in which the substances tested for combination with the enzymes, but not the free enzymes, are sedimented. The enzymes remaining in the supernatant fluids were then estimated quantitatively.

Quantitative estimations of the enzymes. Pepsin and trypsin were incubated, respectively, with acid or slightly alkaline casein solutions for a given period at 37° and the casein which still remained precipitable by 1% trichloroacetic acid was estimated gravimetrically, acid solutions being neutralized before the addition of trichloroacetic acid.

Invertase was incubated with sucrose for a given period at room temperature, and the amount of reduction of Fehling's solution by the reducing sugar formed was estimated by a micro-Bertrand method.

The relative concentrations of the enzymes were calculated by comparing their effects with those of different concentrations of control enzyme solutions incubated simultaneously in the same conditions. If the effects increased with concentrations in a non-linear manner, the calculations were made by graphic interpolation, and if they increased linearly, by the ratio of the effect of tested solutions to that of the control.

In the experimental conditions used in this work amounts of casein rendered soluble in 1% trichloroacetic acid by pepsin increased with increasing concentrations of pepsin in a non-linear manner. On the other hand, there was a direct proportionality between amounts of casein rendered soluble in 1% trichloroacetic acid by trypsin and concentrations of trypsin, and also between amounts of reducing sugar formed from sucrose and concentrations of invertase.

Serological precipitin tests were made as previously described (Kleczkowski, 1943). Increasing dilutions of virus preparations were titrated against constant dilutions of antisera.

Infectivity tests were made by the local lesion method; tobacco mosaic virus was tested on *N. glutinosa*, and potato virus *X* on *N. tabacum* var. White Barley.

EXPERIMENTAL

Experiments with pepsin

Table 1 gives the results of an experiment to test the ability of pepsin at pH 4·0 to combine with suspensions of the two viruses and with a suspension of heat-denatured tobacco mosaic virus.

The suspensions of tobacco mosaic virus were made by precipitating the virus at pH 3·5, centrifuging down the precipitate and resuspending it in pH 4·0 buffer. Potato virus *X* was precipitated by one-quarter saturation with $(NH_4)_2SO_4$ and resuspended in pH 4·0 buffer. The suspension of heat-denatured tobacco mosaic virus was made by resuspending in pH 4·0 buffer the precipitate produced by heating, at 100°, a virus solution for 5 min. at pH 7·0 in the presence of 1% NaCl.

The suspensions were mixed with a pepsin solution and immediately centrifuged for 5 min. at 10,000 r.p.m., and pepsin was estimated in the supernatant fluids. It will be seen that potato virus *X* and heat-denatured tobacco mosaic virus removed most of the pepsin from the solutions, whereas native tobacco mosaic virus did not remove any detectable quantity.

Similarly there was no detectable combination between tobacco mosaic virus and pepsin at pH 3·0 and 3·5, whereas potato virus *X* combined almost as much pepsin at pH 4·5 as at pH 4·0. The method described could not be used over a wider pH range, as the viruses can be sedimented by low speed centrifugation only over a narrow pH range around their isoelectric points.

As heat-denatured tobacco mosaic virus is insoluble over a wide pH range, its combination with pepsin could be tested over a wide pH range. Fig. 1 (curve *A*) shows that maximum combination was at pH about 2·5, the same value as that found by Northrop (1920) for maximum combination between pepsin and coagulated egg albumin. This does not correspond, however, with maximal hydrolysis of heat-denatured tobacco mosaic virus during 1 hr. incubation at 37° (cf. line *B*, Fig. 1).

Table 1. *Combination of pepsin with unchanged and heat-denatured tobacco mosaic virus, and with potato virus X*

	Test mixture		Data for supernatant fluid from centrifugal test mixture*		
Test no.	1 ml. buffer (pH 4·0) containing virus (mg.)	1 ml. H_2O containing pepsin (%)	Precipitate obtained with trichloroacetic acid (mg.)	Casein digested† (mg.)	Pepsin removed by virus‡ (% of total)
	(*a*) *Unchanged tobacco mosaic virus*				
1	4	0·1	8·1	15·4	0
2	2	0·1	8·3	15·2	0
3	1	0·1	8·0	15·5	0
	(*b*) *Denatured tobacco mosaic virus*				
4	4	0·1	15·9	7·6	80
5	2	0·1	10·4	13·1	50
6	1	0·1	9·1	14·4	25
	(*c*) *Potato virus X*				
7	4	0·1	19·0	4·5	90
8	2	0·1	17·8	5·7	85
9	1	0·1	13·0	10·5	65
	(*d*) *Control solutions—no virus*				
10	—	0·1	8·2	15·3	—
11	—	0·05	10·4	13·1	—
12	—	0·025	15·5	8·0	—
13	—	0·012	17·0	6·5	—
14	—	0·006	20·2	3·3	—
15	—	0·0	23·5	—	—

* The mixtures of the viruses with pepsin were immediately centrifuged for 5 min. at 10,000 r.p.m. and supernatant fluids decanted. To 1 ml. of each fluid 2 ml. of 1·2% acid casein were added and the mixtures were incubated for 3 hr. at 37°; they were then neutralized and 0·3 ml. of 10% trichloroacetic acid added to each mixture. The precipitates were centrifuged down, resuspended in 2 ml. H_2O, filtered off, dried and weighed.

† The differences between 23·5 mg. and the amounts precipitated by trichloroacetic acid.

‡ Computed approximately by graphic interpolation from the data of the tests nos. 10–15.

The ability of pepsin to combine with casein, heat-denatured human serum globulin, insoluble starch, kaolin and charcoal was also tested by the same method. The results, given in Table 2, show that casein, globulin and charcoal combined with most of the pepsin, but there was no detectable combination with starch and kaolin.

To see whether pepsin could combine with potato virus *X* without causing any detectable changes, the precipitate of the virus centrifuged from the mixture with pepsin (test no. 7, Table 1) was immediately redissolved in 2 ml. of M/15 phosphate buffer

at pH 7·0. The general appearance of the solution and of its anisotropy of flow, the nitrogen content (micro-Kjeldahl), its serological precipitin titre and its infectivity at 1/1000, did not differ from those of the control preparation. The precipitate of the heat-denatured tobacco mosaic virus, centrifuged from the mixture with pepsin (test no. 4, Table 1), also contained the same amount of nitrogen as a control suspension. Thus it seems that combination of pepsin with potato virus *X* or with heat-denatured tobacco mosaic virus takes place before any further action of the enzyme upon the two substrates becomes noticeable.

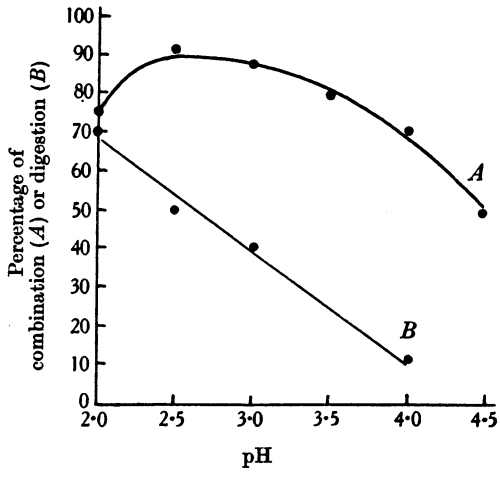

Fig. 1. The effect of pH on combination of pepsin with heat-denatured tobacco mosaic virus and on the rate of digestion during 1 hr. Curve *A*, percentage of pepsin combined. Curve *B*, percentage of virus protein dissolved by pepsin. *Combination.* 1 ml. of buffer solutions of different pH's containing 2 mg. of the denatured virus was mixed with 1 ml. of 0·1% pepsin. Virus suspensions were removed immediately by a short centrifugation, all the supernatants adjusted to the same pH (3·0) and the amounts of pepsin estimated as described in Table 1. *Rate of digestion.* 1·0% suspensions of the denatured virus in buffers of different pH's were incubated for 1 hr. at 37° with 0·15% pepsin. Then the suspensions were centrifuged down and the nitrogen estimated (micro-Kjeldahl).

Table 2. *Combination of pepsin with various substances*

1 ml. of buffer solutions containing suspensions of given substances was mixed with 1 ml. of 0·1% pepsin solution and centrifuged for 5 min. at 10,000 r.p.m. immediately, or after a period of incubation, and the amounts of pepsin in the supernatant fluids estimated as described in Table 1.

Substance tested for combination			Period of incubation (min.)	Pepsin removed (approx. % of total)
Name	Amount (mg.)	pH		
Casein	5·0	4·5	0	90
	2·5	4·5	0	80
	1·2	4·5	0	70
	0·6	4·5	0	50
Denatured human serum globulin	2·5	4·0	20	95
Charcoal	5·0	4·0	20	85
Kaolin	5·0	4·0	20	0
Starch	5·0	4·0	20	0

When a 0·2% suspension of potato virus *X* at pH 4·0 was incubated with 0·05% pepsin for 12 hr. at 37°, the fluid became water-clear and a small resinous mass (nuclein) floated at the surface. Anisotropy of flow, serological activity and infectivity were lost. The fluid did not give a precipitate on one-quarter saturation with $(NH_4)_2SO_4$ or with 1% trichloroacetic acid. A control incubated in the same conditions without pepsin remained apparently unchanged. The precipitate of heat-denatured tobacco mosaic virus, incubated similarly with pepsin for 12 hr., was entirely dissolved, and the resulting water-clear solution did not give a precipitate on one-third saturation with $(NH_4)_2SO_4$ or (after neutralization) with 1% trichloroacetic acid. A similarly treated control incubated without pepsin remained apparently unchanged.

For comparison a 0·2% suspension of native tobacco mosaic virus, at pH 3·0, was incubated with 0·1% pepsin for 10 days at 37°, and then centrifuged down and redissolved in M/15 phosphate buffer at pH 7·0. The general appearance of the solution and of its anisotropy of flow did not differ from those of a control incubated in similar conditions without pepsin, neither was there any difference in the amount of nitrogen, in the serological titre or in infectivity (inoculated at a dilution of 1/1000 into 18 half-leaves of *N. glutinosa* it gave 43 lesions per leaf compared with 39 given by the control).

Experiments with trypsin

To test the ability of trypsin to combine with tobacco mosaic virus and with potato virus *X* at pH 7·0, high-speed centrifugation was used. The viruses were sedimented from mixtures with the enzyme by centrifugation for 1 hr. at 40,000 r.p.m., and the trypsin remaining in the supernatant fluids was then estimated. Table 3 gives the details and results of the experiment; each virus removed some trypsin, but tobacco mosaic virus removed more than potato virus *X*, and the difference became greater with increasing concentration of trypsin.

In tests with pepsin and potato virus *X* at pH 4·0, the virus could be removed from mixtures before undergoing any detectable changes. This could not be done with mixtures of potato virus *X* and trypsin at pH 7·0, as centrifugation necessitated 2½ hr. exposure of the virus to trypsin (1 hr. at the top speed). The pellets of potato virus *X* sedimented from the mixtures with trypsin (tests nos. 1 and 5, Table 3)

Table 3. *Combination of trypsin with potato virus* X *and with tobacco mosaic virus at* pH 7·0

0·1 and 0·025% solutions of trypsin in water (pH 7·0) containing 0·1% of potato virus *X* or tobacco mosaic virus, and controls without the viruses, were centrifuged at 40,000 r.p.m., so that both viruses were sedimented. Trypsin was then estimated in the supernatant fluids. 1 ml. of each supernatant fluid was added to 2 ml. of 1% casein solution (pH 7·3). The mixtures were incubated for given periods of time at 37°, then 0·3 ml. of 10% trichloroacetic acid was added to each mixture; the precipitates were centrifuged down, resuspended in 2 ml. water, filtered off, dried and weighed.

Test no.	Material tested for combination	Conc. of trypsin (%)	Time of incubation (hr.)	Casein precipitated by trichloroacetic acid (mg.)	Casein digested (mg.)	Trypsin removed* (% of total)
1	Potato virus *X*	0·1	3	9·8	10·7	5
2	Tobacco mosaic virus	0·1	3	12·9	7·6	30
3	Trypsin control	0·1	3	9·2	11·3	—
4	None	0·0	3	20·5	—	—
5	Potato virus *X*	0·025	6	16·0	4·5	25
6	Tobacco mosaic virus	0·025	6	16·7	3·8	40
7	Trypsin control	0·025	6	14·6	5·9	—
8	None	0·00	6	20·5	—	—

* As within the tested range of trypsin concentrations and incubation times the amounts of digested casein were directly proportional to trypsin concentrations, percentages of trypsin removed were directly computed from the ratio of casein digested by tested solutions to that digested by the control.

were immediately redissolved in water, precipitated by one-quarter saturation with $(NH_4)_2SO_4$ and redissolved in the original volume of water. Although the general appearance and anisotropy of flow of the preparations did not differ noticeably from those of the control, their infectivity and serological activity were reduced (Table 4).

Although trypsin cannot hydrolyze tobacco mosaic virus, it does combine with the virus, and this offers a possible explanation of reversible inhibition of infectivity. There was too much trypsin in the pellets sedimented from mixtures of the enzyme with tobacco mosaic virus (Table 3, tests nos. 2 and 6) for it to be contained as a solution in the free water of the pellets. This could be seen from the fact that the volume of the pellets was only a small fraction of that of fluids from which they

Table 4. *Infectivity and serological activity of potato virus* X *sedimented by high-speed centrifugation from its mixture with trypsin*

Tested material	Serological titre	Infectivity. Average number of lesions/leaf with material diluted 1/100	1/1000
Pellet from 0·1% trypsin solution (test no. 1, Table 3)	1/320	48	10
Pellet from 0·025% trypsin solution (test no. 5, Table 3)	1/480	51	11
Control	1/640	63	17

were sedimented. Hence most of the trypsin must have been combined with the virus. The experiment shown in Table 5 was made to see how easily this combination could be broken by washing with water. A pellet sedimented by a high-speed centrifugation from a mixture of the virus and trypsin, at pH 7·0, was redissolved in water and the solution was centrifuged again. It will be seen that about one-half of the trypsin was thus extracted from the virus by water, which suggests that the combination is readily reversible.

Table 5. *Removal of trypsin from tobacco mosaic virus by washing at* pH 7·0

15 ml. of a mixture containing 0·05% trypsin and 0·2% tobacco mosaic virus were centrifuged for 1 hr. at 40,000 r.p.m. The pellet was dissolved in water added to make up the volume to 7·5 ml. Half was kept as redissolved *first pellet*, the other half was made up to 7·5 ml. with water, again centrifuged and the pellet dissolved in water added to make up the volume to 3·75 ml. This was described as the dissolved *second pellet*.

Solutions of each pellet, and also solutions of the virus not treated with trypsin, were mixed with double volumes of water or 1·25% casein at pH 7·3. The mixtures were incubated for 6 hr. at 37°, then 0·3 ml. of 10% trichloroacetic acid was added, the precipitates were centrifuged down, resuspended in 2 ml. water, filtered off, dried and weighed.

Material tested for tryptic activity. 1 ml. of solution of	Water added (ml.)	Casein solution added (1 ml. of 1·25%)	Precipitate with trichloroacetic acid (mg.)	Casein digested (mg.)
First pellet	0	2	19·0	10·0
	2	0	3·7	—
Second pellet	0	2	23·5	5·5
	2	0	3·8	—
0·4% tobacco mosaic virus	0	2	28·9	0
	2	0	4·0	—
None	1	2	25·1	—

The ability of trypsin to combine with heat-denatured tobacco mosaic virus at pH 4·0 and 7·0 could be tested by low-speed centrifugation. Tobacco

mosaic virus was denatured by heating for 5 min. at 100° in the presence of 0·5 % NaCl, centrifuged down and resuspended in buffer solutions of the required pH. Equal volumes of 0·1 % trypsin were added, the mixtures were immediately centrifuged, and the trypsin in the supernatant fluids was estimated in the usual way. The results, given in Table 6, show that a proportion of trypsin was combined at both pH values, but considerably more at pH 7·0 than at pH 4·0.

Table 6. *Combination of heat-denatured tobacco mosaic virus with trypsin at* pH 4·0 *and* 7·0

Concentration of virus suspension mixed with trypsin (%)	Percentage of trypsin removed	
	At pH 4·0	At pH 7·0
0·4	20	70
0·2	10	60
0·1	5	55

The ability of trypsin to combine with suspensions of tobacco mosaic virus and potato virus *X* at pH 4·0 was also tested, and compared with the ability to combine with heat-denatured tobacco mosaic virus. The mixtures contained 0·2 % virus suspensions and 0·015 % trypsin. Heat-denatured tobacco mosaic virus combined with about 25 % and native tobacco mosaic virus with slightly less trypsin, whereas potato virus *X* did not combine with any amount detectable by this method.

Experiments with invertase

In a further attempt to assess the specificity of combination of pepsin and trypsin with their substrates, the ability of invertase to combine with the two viruses and with casein was tested. To ensure that combination with amounts of invertase comparable to those of pepsin or trypsin should not be missed, it was desirable that the concentration of invertase used should be the same or smaller than the concentration of pepsin and trypsin. It is believed that this condition was fulfilled by using 0·002 % solution of the invertase preparation for comparison with 0·05 % solutions of pepsin and trypsin, for at these concentrations comparable proportions of the three enzymes were adsorbed by 0·5 % charcoal.

1 ml. of 0·004 % invertase was mixed with 1 ml. of 1 % suspensions of each of the following substances: tobacco mosaic virus in pH 3·5 buffer, heat-denatured tobacco mosaic virus in pH 3·5 buffer, potato virus *X* in pH 4·0 buffer and casein in pH 4·5 buffer. After 90 min. at room temperature the suspensions were centrifuged and invertase estimated in the supernatant fluids. 0·25 ml. of 5 % sucrose solution was added to 1 ml. of each supernatant fluid and after 4 hr. incubation at room temperature the amounts of hydrolyzed sucrose were estimated. In these conditions the amounts of reducing sugar were found to be directly proportional to invertase concentration, so that invertase in the tested solutions could be computed from the ratio of the amount of reducing sugar to that in the control solutions of invertase.

All the supernatant fluids contained the same amounts of invertase as their respective controls, showing that none of the protein suspensions combined with any detectable amount of invertase. In similar conditions 1 % charcoal suspension in pH 4·0 buffer removed 90 % of the invertase.

The ability of invertase to combine with tobacco mosaic virus in solution was also tested at pH 6·5. 0·004 % invertase solution in pH 6·5 M/15 phosphate buffer was mixed with an equal volume of 1 % solution of the virus at the same pH. The virus was then sedimented by centrifugation for 1 hr. at 40,000 r.p.m., and invertase estimated in the supernatant fluid. The supernatant fluid had the same invertase activity as a control solution of invertase not mixed with the virus but otherwise treated similarly. Thus no detectable amount of invertase combined with the virus.

The result of the experiment was checked by testing the pellet sedimented from the mixture of the virus and invertase for the presence of invertase. The pellet was redissolved in the original volume of pH 6·5 buffer, and 2 ml. of the solution was mixed with 0·5 ml. of 5 % sucrose. After incubation for 3 hr. at room temperature the virus was removed from the mixture by adding 0·2 ml. of $\frac{2}{3}$ N-H_2SO_4 and 0·2 ml. of 10 % sodium tungstate. The precipitate was centrifuged off and the supernatant fluid was tested for reducing sugar. None was detected. To exclude the possibility that the presence of the virus might have interfered with enzymic activity of invertase, 2 ml. of 0·5 % solution of the virus in pH 6·5 buffer, containing 0·002 % invertase, was also mixed with 0·5 ml. of 5 % sucrose, incubated simultaneously and then tested similarly. It contained the same amount of hydrolyzed sucrose as a control solution containing only invertase and sucrose.

Some effects of adsorption of the enzymes by charcoal. Extraction of the enzymes from charcoal. Protection of trypsin by tobacco mosaic virus from spontaneous inactivation at pH 7·0

Hedin (1906) found that the addition of sufficient charcoal to adsorb almost all the trypsin in a solution reduced its enzymic activity, and that this reduction of activity increased with the length of time the charcoal and the enzyme were in contact before the substrate (casein) was added. That most of the trypsin was adsorbed could be shown by filtering

off the charcoal before adding casein, when the filtrate showed almost no tryptic activity. Table 7 shows that the activity of trypsin adsorbed on charcoal steadily decreased during the period between adding charcoal and casein. This loss of activity is probably due to denaturation of trypsin on the surface of the charcoal.

Table 7. *The effect of incubating trypsin with charcoal at* pH 7·0

1 vol. of 1·5% water-suspension of charcoal was added to 2 vol. of 0·06% trypsin solution. The mixture was incubated for given lengths of time at room temperature and then 6 vol. of 1% casein (pH 7·0) were added, and the mixture incubated for 3 hr. at 37°. In one group of experiments charcoal was removed (by centrifugation) before casein was added, and in the other it was not removed. The amounts of digested casein were then estimated as in Table 3. Charcoal was removed immediately before the estimation.

Time of incubation of trypsin-charcoal mixture (min.)	Treatment	Casein digested (mg. in 3 ml. of mixture)	Tryptic activity remaining (% of total)*
0	Charcoal removed before casein was added	2·2	21
5		2·0	19
15		1·8	17
60		1·2	11
0	Charcoal not removed	8·8	84
5		6·7	64
15		5·1	49
60		2·0	19
—	Control (water added instead of charcoal suspension)	10·5	—

* Computed from the ratio of casein digested by a tested sample to that digested by the control (see Table 3).

A similar experiment with pepsin (with the same concentrations of the reagents in buffer at pH 4·0) showed that the activity of pepsin adsorbed on charcoal also steadily decreased during the period between adding charcoal and casein, but the rate of the decrease was much slower than with trypsin. The fall in the activity after 24 hr. was approximately equal to that of trypsin after 30 min.

Although tobacco mosaic virus combines with trypsin, the presence of the virus has no effect on the ability of the enzyme to hydrolyze casein. Indeed, the presence of the virus protects trypsin from the spontaneous inactivation which it normally undergoes during incubation at pH 7·0. This protective effect of the virus is shown by the experiment described in Table 8. One-half of the tryptic activity of a trypsin solution was destroyed during 24 hr. at 37°, whereas only one-fifth was lost in the presence of the virus.

Table 8. *Protection of trypsin by tobacco mosaic virus*

0·1% trypsin solution was mixed with an equal volume of 1% tobacco mosaic virus or with water and the mixtures incubated for 24 hr. at 37°. Then 4 vol. of 1% casein solution (pH 7·0) were added to each mixture and the mixtures were incubated for 4 hr. at 37°. Amounts of digested casein were then estimated as described in Table 3.

Treatment of trypsin before incubation with casein	Casein digested (mg. in 3 ml. of mixture)	Tryptic activity remaining (% of total)
Mixture trypsin + virus incubated for 24 hr. at 37°	15·25	80
Trypsin and virus incubated separately for 24 hr. at 37° and then mixed	9·5	50
Trypsin + water incubated for 24 hr. at 37°	9·8	52
Trypsin freshly dissolved	18·9	100

Table 9. *Extraction of trypsin from charcoal by casein*

0·15% trypsin solutions were mixed with equal volumes of 1·5% charcoal suspensions in water or with equal volumes of water (control). 4 vol. of 1% casein were added, and the mixtures were incubated at 37°. Charcoal was removed by centrifugation at given times. Amounts of digested casein were estimated as described in Table 3 after 1 hr. and after 3 hr. incubation.

Test no.	Time at which charcoal was removed	Casein digested (mg. in 3 ml. of mixture) after 1 hr.	Casein digested (mg. in 3 ml. of mixture) after 3 hr.
1	Before adding casein	—	2·0
2	Immediately after adding casein	—	6·2
3	1 hr. after adding casein	4·3	10·3
4	At the end of incubation with casein	4·3	11·5
5	Control (no charcoal added)	9·4	16·0

Hedin (1907) showed that casein can set free trypsin adsorbed on charcoal. The experiment described in Table 9 confirms this. It can be seen that the removal of charcoal from a trypsin solution, immediately after adding casein, reduced tryptic activity of the solution much less than if the charcoal was removed before adding casein. After incubation for 1 hr. at 37°, casein liberated most of the trypsin, for removing the charcoal then had little influence on subsequent hydrolysis of casein. Since casein liberated the adsorbed trypsin, it was impossible to determine directly whether trypsin was active while adsorbed on charcoal.

When charcoal was added to a solution of 0·1% trypsin already containing 1% casein, the presence of casein entirely prevented adsorption of the trypsin by charcoal. The presence of 1% tobacco mosaic

virus in 0·1% trypsin could not prevent the adsorption, though it considerably reduced the amount of trypsin adsorbed. However, when the virus was added to a solution of trypsin already containing charcoal, it could not liberate any trypsin already adsorbed on charcoal.

can hydrolyze sucrose, although the rate of hydrolysis was somewhat slower than with free invertase. That the enzyme remained attached to charcoal was shown by the fact that at any time during incubation the hydrolysis could be stopped by removing the charcoal.

Table 10. *Extraction of invertase from charcoal by casein at* pH 6·0

3 ml. of 0·0025% invertase solution in pH 6·0 buffer were mixed with 0·3 ml. of 5% charcoal in the buffer or with 0·3 ml. of the buffer, and the mixtures incubated for 1½ hr. at room temperature. 0·3 ml. of 5% casein in the buffer, or 0·3 ml. of the buffer, were added immediately after charcoal or at the end of the incubation period. At the end of the incubation period enough 10% sucrose was added to give 1% sucrose solution, and the mixtures were incubated for 4 hr. at room temperature. Charcoal was removed by centrifugation at given times. At the end of the incubation 1/10 vol. of ⅔ N-H_2SO_4 and of 10% sodium tungstate were added to all solutions, the precipitates of casein were removed by centrifugation, and 1 ml. of each solution was assayed for reducing activity.

Time at which casein was added	Time at which charcoal was removed	Reducing power of solution (mg. Cu/ml.)
None added	Immediately before adding sucrose	0·84
None added	Immediately after adding sucrose	0·89
Immediately after charcoal	Immediately before adding sucrose	3·01
1½ hr. after charcoal	Immediately before adding sucrose	2·37
None added	At the end of the incubation with sucrose	2·56
None added	No charcoal added (control)	3·20

Table 11. *Activity of invertase adsorbed on charcoal*

1 vol. of 5% charcoal suspension was added to 9 vol. of 0·002% invertase solution in pH 6·0 buffer, and the mixture incubated at room temperature for 1½ hr. Then enough 12% sucrose was added to give 1% sucrose solution. Charcoal was removed by centrifugation at given times, and 1 ml. of each solution assayed for reducing activity after 1½ and 4 hr. incubation with sucrose.

Time at which charcoal was removed	Reducing activity (mg. Cu/ml.) after 1½ hr.	Reducing activity (mg. Cu/ml.) after 4 hr.
Immediately before adding sucrose	0·06	0·16
Immediately after adding sucrose	0·08	0·20
1½ hr. after adding sucrose	0·83	0·85
At the end of incubation with sucrose		2·30
Control (buffer added instead of charcoal suspension)	1·10	2·94

The experiment described in Table 10 shows that casein solution at pH 6·0 could also remove invertase from charcoal, whereas sucrose could not (see also Table 11). Human serum albumin and tobacco mosaic virus also extracted invertase from charcoal, although albumin was less effective than casein, and the virus less than albumin. The extraction of an enzyme from charcoal, therefore, is clearly no indication that the substance responsible for the extraction is a substrate or that it can even combine with the enzyme.

Nelson & Griffin (1916) found that invertase adsorbed on charcoal hydrolyzed sucrose at the same rate as did the free enzyme. The experiment described in Table 11 confirms that adsorbed invertase

DISCUSSION

It is clear from the results described in this paper that no general statement can be made about the specificity of forces leading to combination between proteolytic enzymes and their substrates, for individual enzymes differ in their behaviour. The experiments with pepsin all suggest that there are such forces, as this enzyme combined with all the substrates used but did not combine with tobacco mosaic virus. When this virus was turned into a substrate as a result of denaturation by heat, combination could then take place. Thus details of structure of proteins that determine their susceptibility to proteolytic activity of pepsin also seem to determine the ability of pepsin to combine with proteins. This, however, is not true of trypsin, which combined with all the proteins tested. Indeed, more trypsin entered into combination with tobacco mosaic virus, which is not a substrate for its proteolytic activity, than with potato virus *X*, which is a substrate. This combination does not seem to be simple adsorption of the enzyme by the virus, for tobacco mosaic virus did not combine with pepsin and neither virus combined with invertase. By contrast, charcoal, which presumably combined by surface adsorption, combined with all three enzymes and with all the other proteins used. The liberation of enzymes from charcoal by proteins cannot be considered as evidence of their affinity to the enzymes, for it can also occur with enzymes for which the proteins are not substrates, and with which they do not combine. The liberation is most

likely a result of replacement of one adsorbed protein by another.

The immediate inhibition of infectivity when trypsin is added to solutions of some plant viruses has been interpreted differently by different authors. Stanley (1934*a*) concluded from the results of his infectivity experiments with tobacco mosaic virus that trypsin was affecting the susceptibility of the host plant and not the virus. He found no difference between the rate of diffusion of trypsin in the presence and absence of the virus and took this as evidence that there was no combination. Bawden & Pirie (1936) suggested that the virus and trypsin might combine to give a non-infective complex, which breaks down on dilution; they pointed out that Stanley's diffusion experiments might have been made with such an excess of trypsin that no differences in the diffusion rate could have been detected even if such a complex were formed. Stanley himself took this possibility into account, and, therefore, did not consider his evidence as a proof of lack of combination. Since then, Hills & Vinson (1938) have found that the diffusion rate of both trypsin and the virus are influenced when the two are present in a solution together. The experiments described here do not exclude the possibility that trypsin affects the susceptibility of the host plant. However, they do show that trypsin combines with tobacco mosaic virus and with potato virus *X*, and that the combination is fairly readily broken by dilution in water, and it seems most reasonable that the union of trypsin with the viruses leads to inhibition of infectivity.

A similar effect has been described by Loring (1942) with ribonuclease and tobacco mosaic virus. Ribonuclease, which decomposes nucleic acid separated from the denatured virus, has no effect on the intact virus, but it combines with it and causes an inhibition of infectivity. Trypsin and ribonuclease have two features in common that might account for their ability to combine with tobacco mosaic virus. Both are able to decompose parts of the denatured virus, so that there may be some specific attraction between these parts of the intact virus and the enzymes, even though some structural details prevent enzymic decomposition. Secondly, both enzymes have isoelectric points near pH 7·0, whereas the isoelectric point of the virus is at pH 3·3, so that their combination with the virus could result from differences of charge.

Mere combination of potato virus *X* with pepsin has no effect on infectivity, which suggests that different parts of the virus particle are involved in combination with pepsin and trypsin. As combination between tobacco mosaic virus and trypsin protects trypsin from spontaneous inactivation at pH 7·0 and also causes inhibition of infectivity of the virus, the combination seems to involve parts of the virus essential for infectivity, on the one side, and active parts of the enzyme, on the other side.

SUMMARY

1. Pepsin combines with potato virus *X* and casein, which are substrates for its proteolytic activity, but not with tobacco mosaic virus, which is not a substrate.

2. Tobacco mosaic virus denatured by heat is readily hydrolyzed by pepsin and combines with pepsin almost to the same extent as potato virus *X*.

3. Invertase does not combine with potato virus *X*, with tobacco mosaic virus, whether heat-denatured or not, or with casein.

4. More trypsin combines with tobacco mosaic virus, which is not a substrate for its proteolytic activity, than with potato virus *X*, which is a substrate. The combination of trypsin with tobacco mosaic virus could account for the reversible inhibition of infectivity of the virus by trypsin.

5. Combination between trypsin and tobacco mosaic virus protects trypsin from spontaneous inactivation at pH 7·0.

6. Trypsin and invertase adsorbed on charcoal can be set free by casein; invertase can also be extracted by tobacco mosaic virus, but not by sucrose.

I wish to thank Mr N. W. Pirie for doing all the high-speed centrifugations.

REFERENCES

Bawden, F. C. & Pirie, N. W. (1936). *Brit. J. exp. Path.* **17**, 64.
—— —— (1937). *Proc. Roy. Soc.* B, **123**, 274.
—— —— (1938). *Brit. J. exp. Path.* **19**, 66.
—— —— (1943). *Biochem. J.* **37**, 66.
Bayliss, W. M. (1906). *Biochem. J.* **1**, 175.
Caldwell, J. (1933). *Ann. appl. Biol.* **20**, 100.
Hedin, S. G. (1906). *Biochem. J.* **1**, 484.
—— (1907). *Biochem. J.* **2**, 81.
Hills, C. H. & Vinson, C. G. (1938). *Res. Bull. Mo. agric. Exp. Sta.* No. 286.
Kleczkowski, A. (1943). *Biochem. J.* **37**, 30.
Loring, H. S. (1942). *J. gen. Physiol.* **25**, 497.
Nelson, J. M. & Griffin, E. G. (1916). *J. Amer. chem. Soc.* **38**, 1109.
Northrop, J. H. (1920). *J. gen. Physiol.* **2**, 113.
Ross, A. F. & Vinson, C. G. (1937). *Res. Bull. Mo. agric. Exp. Sta.* No. 258.
Stanley, W. M. (1934*a*). *Phytopathology*, **24**, 1055.
—— (1934*b*). *Phytopathology*, **24**, 1269.